Cytochrome c Protein and Assay

Technical Field

The present invention relates to a cytochrome c-reporter fusion protein construct which targets the mitochondria and has a reduced ability to induce apoptosis in a living cell. The fusion construct of the invention can be used in assays for detecting early events in apoptosis in living cells.

Background to the Invention

'Programmed cell death' or apoptosis is a key event in multicellular organisms, defining a genetically encoded cell death program which is morphologically, biochemically and molecularly distinct from necrosis (Vermes et al., J Immunol Meth., (2000) 243, 167-190). The characteristic morphological signs of apoptosis (cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation) are the results of a complex biochemical cascade of events which is an integral part of physiological homeostasis.

Apoptosis ensures an equilibrium between cell proliferation and cell death, thus playing a regulatory role in the control of the size of cell populations and tissues. Aberrations in cell death signalling, in membrane or cytoplasmic receptors, or alterations in genes that govern apoptosis are involved in the pathogenesis of congenital malformations and many acquired diseases (Haanen & Vermes, Eur J Obstetr.Gynecol., (1996) 64, 129-133). Too little apoptosis may result in malignancies (Tomlinson & Bodmer, Proc. Natl. Acad.Sci. USA, (1995) 92, 11130- 11134), Leukemias (Sachs, Proc. Natl. Acad. Sci. USA, (1996) 93, 4742- 4749) or the resistance to anticancer therapy (Pahor et al., Lancet, (1996) 348, 493-497). Too much apoptosis can result in immune deficiency (Meyaard et al., Science (1992) 257, 217-219) and degenerative conditions (Griffith et al., Science, (1995) 270, 1189-1192).

There is therefore considerable interest within the medical, pharmaceutical and toxicological sciences in developing a greater understanding of the events which trigger and regulate apoptosis. Furthermore, there is a need to develop new techniques which can be used to identify, quantify and characterise agents which can modulate this phenomenon.

Assays for Detecting Apoptosis

A large number of assays have been developed to detect the onset of programmed cell death (Sgone & Wick, Int Arch Allergy Immunol., (1994) 105, 327-332; Sgone & Gruber, Exp Gerontol., (1998) 33, 525-533). These assays are based upon a wide range of events associated with cell death and have traditionally included light and electron microscopy with vital staining and nuclear dyes. Biochemical methods are often employed, for example based upon DNA laddering or degradation, DNA end labelling techniques (e.g. TUNEL –terminal deoxynucleotide transferase dUTP Nick End labelling), nuclease activity and lactate dehydrogenase enzyme release.

Flow cytometry tends to be the most widely used method (Vermes et al., J Immunol Methods, (2000) 243, 167-190) for detecting and quantifying apoptosis because it is amenable to screening large numbers of cells. This fluorescence - based technique employs vital dyes, antibodies to apoptotic enzymes (e.g. caspases) and single stranded DNA breaks, together with probes for measuring calcium flux and phospholipid redistribution.

Flow cytometry allows *in vivo* analysis of cells in suspension, one at a time, at rates of 1000 to 10,000 cells/s. However, one problem with flow cytometry is that it can only be used in cells in suspension, such as liquid cell cultures and cells derived from the hemopoietic system. Furthermore flow cytometry of tissue cells requires physical and enzymatic manipulation to get the cells in suspension, which by itself may trigger apoptosis, necessitating checks by conventional light or fluorescence microscopy.

Existing methods are thus based upon late events in apoptosis (e.g. DNA degradation, caspase assays) and many require cellular fixation and staining with specific antibodies/dyes. None of the techniques described above provide a homogeneous living cell assay, based upon the early events in apoptosis, in real time nor discriminate from cellular necrosis. Furthermore, none of these assays are amenable to high throughput live cell screening which is required to test large numbers of compounds for their ability to modulate apoptosis.

Cytochrome c Translocation as a Marker for Apoptosis

Cytochrome c is a nuclear encoded protein which is targeted to the mitochondria where it performs its biological function as an electron carrier. The translocation of cytochrome c from the mitochondria to the cytoplasm in response to apoptotic stimuli is an early and critical step in the commitment of the cell to undergo apoptosis (Li et al., Cell (1997) 91, 479-489). Cytochrome c binds strongly to apoptosis protease activation factor -1 (Apaf-1) in the cytosol (Zou et al., Cell (1997) 90, 405-413). In the presence of cofactors the resulting cytochrome c: Apaf-1 assembles into a multimeric 'apoptosome' that binds and activates a protease zymogen, procaspase-9 (Srinivasula et al., Mol. Cell (1998) 1, 949-957). This results in the activation of the 'caspase cascade' whereby many intracellular substrates are cleaved disabling important cellular processes and breaking down structural components of the cell (Slee et al., J Cell.Biol. (1999) 144, 281-292; Skulachev, FEBS Lett., (1998) 423, 275-280). A schematic diagram illustrating the mitochondrial role in apoptosis is shown in Figure 1.

Recent *in vitro* studies (Kluck et al., J. Biol. Chem., (2000), 275, 16127-16133; Yu et al., J.Biol.Chem., (2001), 276, 1304-13038) have identified the molecular determinants involved in the cytochrome c: Apaf-1 interaction. Horse cytochrome c has been shown to be highly homologous to human cytochrome c and can initiate caspase activation, whereas yeast cytochrome c did not measurably bind to Apaf-1 nor activate caspase (Yu et al., J Biol Chem., (2001) 276, 13034-13038). These studies focussed on the key differences between horse and yeast cytochrome c and used site directed mutagenesis to generate mutant variants which were subsequently analysed with respect to their ability to activate caspase. The results from this *in vitro* work indicated that residue 7, 25, 39, 62-65 and 72

were critical amino acids for cytochrome c:Apaf-1 interaction. Notably the mutation K72A showed no detectable binding or caspase-9 activation. While the mutation of lysine 72 to alanine abolished the interaction between cytochrome c and Apaf1, respiratory function of cytochrome c was unaffected.

Although the authors demonstrated reduced binding to Apaf-1 and caspase-9 activation *in vitro* it is not known whether the cytochrome c mutant proteins would behave in a similar manner in living cells, targeting the mitochondria and not inducing apoptosis.

Recent reports by Abdullaev et al. (Biochem J. (2002) 362, 749-754), again based on *in vitro* experiments, indicate that the horse K72 mutants described by Yu et al. (J Biol Chem., (2001) 276, 13034-13038) show the same level of caspase activation as the wild-type protein if present at 2-12 fold higher concentrations than the wild type protein. In contrast, the authors reported that a horse K4E cytochrome c mutant was inactive in activating caspase and conclude that this mutant, rather than K72 mutants, would be a good candidate for *in vivo* knock-in studies on the role of cytochrome c in apoptosis.

Fluorescent Proteins

The use of Green Fluorescent Protein (GFP) derived from *Aequorea victoria* is now well known for research into many cellular and molecular-biological processes. Cytochrome c-GFP fusions have been used in studies on apoptosis. Thus Heiskanen et al. (J Biol Chem., (1999) 274, 5654-5658) expressed a cytochrome c-GFP fusion, based upon rat cytochrome c, in rat pheochromocytoma-6 (PC6) cells. Induction of apoptosis by staurosporine led to release of the fusion from the mitochondria which was accompanied by mitochondrial depolarisation.

Goldstein et al., (Nat Cell Bio., (2000) 2, 156-160) demonstrated mitochondrial localisation of a cytochrome c–GFP fusion, based upon human cytochrome c, over-expressed in HeLa cells. A range of apoptotic inducers were shown to cause rapid release of cytochrome c-GFP.

Other researchers have also used cells which have been transiently transfected to over-produce cytochrome c-GFP fusion proteins to investigate apoptosis. Thus Goa et al., (J. Cell Sci., (2001) 114, 2855-2862) monitored the dynamic redistribution of GFP-tagged cytochrome c and the morphological changes of mitochondria within living HeLa cells during UV-induced apoptosis. Similarly, Lim et al., (J Biomed Sci (2002); 9,488-506) explored the biochemical basis of cytochrome c-GFP localisation by transiently transfecting COS-7 cells with various GFP constructs and determining sub-cellular distribution using fluorescence and immunochemical techniques.

Problem to be Addressed

There is a need to develop sensitive assays which are amenable to high throughput screening in living cells and which allow detection and analysis in real time of the early events in apoptosis.

While over-expression of cytochrome c in cells has been demonstrated, the resulting high levels of protein lead to apoptosis and cell death, necessitating the use of complex inducible/transient systems for studies involving up-regulation of cytochrome c levels (Chandra et al., J Biol Chem. (2002) 277, 50842-50854). To date, it has not been possible to generate stable cell lines over-expressing this protein which could be reliably used for screening purposes.

There is thus a need for a cytochrome c protein which, when coupled to a reporter group to form a fusion construct, can be expressed in cells to provide stable cell lines. The resulting construct can act as a biosensor within the cells for early events in the induction or repression of apoptosis. Stable cell lines overexpressing such constructs are suitable for high throughput screening purposes to identify agents which modulate apoptosis.

The present invention addresses this problem and provides a fusion construct which has no observable toxicity to cells and acts as a biosensor to permit the detection of early events in apoptosis in living cells. The stably transformed cells of the invention can still undergo apoptosis due to the presence of endogenous cytochrome c, thus allowing

detection and determination of a second apoptotic signalling event whether up or downstream of the cytochrome c tranlsocation.

Summary of the Invention

In a first aspect of the present invention there is provided a cytochrome c-reporter fusion protein construct comprising a modified cytochrome c protein or any functional analogue thereof derived from wild type cytochrome c, wherein the modified cytochrome c targets the mitochondria and has a reduced ability to induce apoptosis in a living cell.

A reporter is to be understood to be any group that is detectable due to its radioactive, fluorescent or luminescent properties or is localisable by a detectable moiety such as a labelled antibody or specific binding compound.

Preferably, the modified cytochrome c binds apotosis protease activation factor-1 (Apaf-1) at least 10 times less than wild type cytochrome c. More preferably, the modified cytochrome c binds Apaf-1 at least 100 times less than wild type cytochrome c. Most preferably, the modified cytochrome c binds Apaf-1 at least 1000 times less than wild type cytochrome c.

Suitably, at least one of the amino acids of the modified cytochrome c at positions 4, 7, 8, 25, 39, 62, 63, 64, 65 and 72 has been mutated relative to the wild type cytochrome c.

Suitably, the modified cytochrome c has an amino substitution or substitutions selected from the group consisting of K4E, K72A, K72L, K72R, K72G, K72X, E62N, K7E-K8E, K25P-K39H, K7A-E62N-K25P, K7A-E62N-K39H, K7E-K8E-E62N, K7A-K25P-E62N, K7A-E62N-K25P-K39H, E62N-T63N-L64M-M65S, K7E-K8E-E62N-K25P-K39H, K7E-K8E-K25P-E62N-T63N-L64M-M65S, K7E-K8E-K39H-E62N-T63N-L64M-M65S and K7E-K8E-K25P-K39H-E62N-T63N-L64M-M65S.

Preferably, the modified cytochrome c comprises the amino acid substitution selected from the group consisting of K7E-K8E-E62N-K25P-K39H, K7E-K8E-K25P-E62N-T63N-L64M-

M65S, K7E-K8E-K39H-E62N-T63N-L64M-M65S and K7E-K8E-K25P-K39H-E62N-T63N-L64M-M65S.

More preferably,the modified cytochrome c comprises the amino acid substitution selected from the group consisting of K72A, K72L, K72R, K72G and K72X, wherein X represents trimethylation. Most preferably, the modified cytochrome c comprises the amino acid substitution K72A or K72L.

Preferably, modified cytochrome c comprises the amino acid substitution K4E.

Suitably, the reporter is a fluorescent protein or a functional analogue thereof.

It will be understood by the person skilled in the art that a functional analogue of a fluorescent protein will include, but is not limited to, any detectable fluorescent protein fragment formed in a protein fragment complementation assay as described, for example, in US 6,270,964, US 6,428,951 and US 6,294,330.

Preferably, the fluorescent protein of the present invention is a Green Flourescent Protein (GFP) derived from *Aequoria Victoria*, *Renilla reniformis* or other members of the class *Anthozoa* (Labas et al., Proc.Natl.Acad.Sci, (2002), 99, 4256-4261).

US 6172188 describes variant GFPs wherein the amino acid in position 1 preceding the chromophore has been mutated to provide an increase in fluorescence intensity. These mutants result in a substantial increase in the intensity of fluorescence of GFP without shifting the excitation and emission maxima. F64L-GFP has been shown to yield an approximate 6-fold increase in fluorescence at 37 ^{o}C due to shorter chromophore maturation time.

One mutant, commonly termed Enhanced Green Fluorescent Protein (EGFP), contains the mutations F64L and S65T (Cormack, B.P. et al., Gene, (1996), 173, 33-38). EGFP has been optimised for expression in mammalian systems, having been constructed with preferred mammalian codons.

Suitably, the fluorescent protein is selected from the group consisting of Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP), Blue Fluorescent Protein (BFP), Cyan Fluorescent Protein (CFP), Red Fluorescent Protein (RFP), Enhanced Green Fluorescent Protein (EGFP) and Emerald. Preferably, the fluorescent protein is either EGFP or Emerald.

GB 2374868 describes GFP derivatives having a triple mutation at F64, S65/E222 and S175 which exhibit enhanced fluorescence relative to wild type GFP when expressed in non-homologous cells at temperatures above 30^{O}C and when excited at about 490 nm. Mutant GFPs produced using the method of the invention provide a means for detecting GFP reporters in mammalian cells at lower levels of expression and/or increased sensitivity relative to wild type GFP.

Preferably, the GFP of the present invention comprises

i) an amino acid substitution at position F64L;
ii) an amino acid substitution at position S175G; and
iii) an amino acid substitution at position E222G.

In a preferred embodiment the fusion construct is either SEQ ID NO: 4 or SEQ ID NO: 6.

In one embodiment, the reporter is localisable by a detectable luminescent, fluorescent or radio-active moiety. Thus, for example, the reporter comprises an immunogenic motif and the detectable moiety may be a luminescent, fluorescent or radio-actively labelled antibody.

Suitably, the reporter comprises a FLAG™, HA, HIS, c-Myc, VSV-G, V5 or a HSV (Sigma-Aldrich) epitope which is localisable by specific labelled antibodies.

In another embodiment, the reporter comprises a cysteine-rich motif and the detectable moiety comprises a labelled biarsenical compound as described by Griffin et al., Science (1998), 281, 269-272) and in US 6,054,271, US 6,008,378 and US5,932,474.

In a second aspect of the present invention, there is provided a nucleotide sequence encoding a protein fusion construct as hereinbefore described.

Preferably the nucleotide sequence is SEQ ID NO: 3 or SEQ ID NO: 5.

In a third aspect of the present invention, there is provided a nucleic acid construct comprises a suitable control region and the nucleotide sequence as hereinbefore described, the sequence being under the control of the control region.

Suitably, the nucleic acid construct is under the control of a promoter selected from the group consisting of native cytochrome c promoter, mammalian constitutive promoter, mammalian regulatory promoter, human ubiquitin C promoter, viral promoter, SV40 promoter, CMV promoter, yeast promoter, filamentous fungal promoter and bacterial promoter.

Preferably, the promoter is the CMV or the SV40 promoter. More preferably, the promoter is the human ubiquitin C promoter.

In a fourth aspect of the present invention, there is provided a replicable vector comprising a nucleic acid construct as hereinbefore described.

Suitably, the vector is a plasmid vector as described by Makrides (Prot Expression & Purif. (1999) 17, 183-202).

Preferably the vector is a viral vector. Suitable viral vectors for use in the invention are described, for example, by Ng et al., Hum Gene Ther. (2000) 11, 693-699 and include cytomegalovirus, Herpes simplex virus, Epstein-Barr virus, Simian virus 40, Bovine papillomavirus, Adeno-associated virus, Adenovirus, Vaccina virus and Baculovirus vector.

In a fifth aspect of the present invention, there is provided a host cell stably transformed with a nucleic acid construct as hereinbefore described.

In a sixth aspect of the present invention, there is provided a host cell transiently transformed with a nucleic acid construct as hereinbefore described.

Suitably, the host cell is selected from the group consisting of plant, insect, nematode, bird, fish and mammalian cell. Preferably the cell is a human cell. More preferably the human cell is selected from the group consisting of Hek, Hela, U2OS and MCF-7. Most preferably the cell is Hek cell line 293 (Hek293).

Suitably, the host cell is capable of expressing the fusion protein as hereinbefore described.

In a seventh aspect of the present invention, there is provided a method for detecting apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct as hereinbefore described;

ii) determining the localisation of the fusion construct within the cell with time;

wherein a change in localisation of the fusion construct within the cell is indicative of apoptosis.

In an eighth aspect of the present invention, there is provided a method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct as hereinbefore described;

ii) determining the localisation of the construct within the cell;

iii) treating the cell with the agent and determining the localisation of the construct within the cell;

wherein any difference in the localisation of the construct within the cell relative to control cells untreated with the agent is indicative of the effect the agent has upon modulating apoptosis.

In a ninth aspect of the present invention, there is provided a method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a first cell and a second cell which both over-express a fusion construct as hereinbefore described;

ii) treating the first cell with the agent and determining the localisation of the construct within the first cell;

iii) determining the localisation of the construct within the second cell which has not been treated with the agent;

wherein any difference in the localisation of the construct within the first cell and second cell is indicative of the effect the agent has upon modulating apoptosis.

In a tenth aspect of the present invention, there is provided a method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct as hereinbefore described;

ii) treating the cell with the agent and determining the localisation of the construct within the cell;

iii) comparing the localisation of the construct in the presence of the agent with a known value for the localisation of the construct in the absence of the agent;

wherein any difference in the localisation of the construct within the cell in the presence of the agent and the known value in the absence of the agent is indicative of the effect the agent has upon modulating apoptosis.

Suitably, the known value according is stored on a database, such as an electronic or optical database.

Suitably, the localisation of the protein fusion is measured by its luminescence, fluorescence or radioactive properties.

The method of the invention is suitable for screening purposes to identify agents which induce or inhibit apoptosis.

In another embodiment of the method of the invention, the localisation of the fusion construct is determined on non-living, fixed cells. Thus, the living cells over-expressing the fusion construct are fixed at specific time points by conventional means and the location of the fusion protein detected using a detectable moiety, such as a labelled antibody or specific binding chemical. In this way, the effect an agent has upon apoptosis can be

determined by comparing localisation of the fusion construct in the presence and the absence of the agent. Alternatively, the effect that the agent has upon apoptosis can be determined by comparing localisation of the construct in the presence of the agent against a known value (for example, one stored on a database) for localisation in the absence of the agent.

Suitably, the agent is a chemical, physical or biological agent. Examples of chemical agents include inorganic and organic compounds, such as drugs, toxins, peptides, proteins and nucleic acids. Physical agents include electromagnetic radiation such as electrical, magnetic and light (UV, gamma, IR, visible) energy. Examples of typical biological agents include viruses, prions, bacteria and fungi which could infect a living cell and modulate apoptosis.

Brief Description of the Drawings

Figure 1. Schematic representation depicting the mitochondrial role in apoptosis; reproduced by kind permission of Biocarta: (www. biocarta.com/ pathfiles/ h_mitochondriaPathway.asp).

Figure 2 a) InCell Analyzer 3000 images (ca. 40x magnification) showing cytochrome c (K72A-GFP mutant) labelled mitochondria in Hek 293 cells of clone 2B6.

Figure 2 b) Confocal microscopic image (ca. 60x) cytochrome c-GFP expression in HeLa cells (from Goldstein et al., (Nat Cell Bio., (2000) 2, 156-160))

Figure 3 a)-c). InCell Analyzer 3000 images showing colocalisation of cytochrome c (K72A mutant)-GFP and Tetramethylrhodamine ethyl ester (TMRE) using dual excitation of cytochrome c and TMRE in Hek 293 cells.

Figure 3 a) Cytochrome c (K72A)-GFP

Figure 3 b) TMRE labelled mitochondria

Figure 3 c) overlay of Fig 3a) & 3b) following dual excitation

Figure 4. Nucleic acid sequence encoding wild type cytochrome c (SEQ ID NO: 1)

Figure 5. Amino acid sequence of wild type cytochrome c (SEQ ID NO: 2)

Figure 6. Nucleic acid sequence encoding F64L-S175G-E222G-triGFP–cytochrome c (K72A) construct (SEQ ID NO: 3)

Figure 7. Amino acid sequence of F64L-S175G-E222G-triGFP–cytochrome c (K72A) construct (SEQ ID NO: 4)

Figure 8. Nucleic acid sequence encoding cytochrome c (K72A)- F64L-S175G-E222G-triGFP (SEQ ID NO: 5)

Figure 9. Amino acid sequence of cytochrome c (K72A)- F64L-S175G-E222G-triGFP (SEQ ID NO: 6)

Figure 10. Nucleic acid sequence encoding F64L-S175G-E222G-wild type cytochrome c construct (SEQ ID NO: 7)

Figure 11. Amino acid sequence of F64L-S175G-E222G wild type cytochrome c construct (SEQ ID NO: 8)

Figure 12. Nucleic acid sequence encoding wild type cytochrome c-F64L-S175G-E222G tri GFP (SEQ ID NO: 9)

Figure 13. Amino acid sequence of wild type cytochrome c-F64L-S175G-E222G tri GFP (SEQ ID NO: 10)

Specific Examples

Example 1

Amplification of the cytochrome c gene, fusion to GFP (F64L-S175G-E222G) and introduction of the K72A (APAF-1 binding) mutation.

The fluorescent-cytochrome c mutant fusion proteins of the current invention were produced by joining, in frame, a sequence of the nucleic acid that encodes for the cytochrome c protein to a sequence of the nucleic acid that encodes for a fluorescent protein and then introducing the K72A (APAF- 1 binding) mutation (Kluck et al., J Biol Chem., (2000) 275, 16127-16133)) to the nucleic acid of the fusion construct. A preferred sequence of the human cytochrome c gene is described by Zang and Gerstein (Gene, (2003) 312, 61-72); NCBI Accession number NM_018947. (SEQ ID NO: 1) the encoded protein is shown in SEQ ID NO: 2. Alternative human cytochrome c sequences may be used. In addition, alternative sequences around the start and stop codons of the gene may be used to provide useful restriction enzyme sites for protein fusion. Where such alterations change the amino acid numbering relative to the reference sequence such numbering should be inferred by amino acid alignment with the reference sequence. Preferred sequences of the gene encoding the fluorescent protein include those derived from *Aequorea victoria* published by Chalfie *et al*, (Science, (1994) 263, 802-5), the GFP-F64L-S175G-E222G mutant (GB Patent 2374868), Emerald (Aurora biosciences), EGFP and related mutants (BD Clontech, Palo Alto, CA), and fluorescent proteins from species of *Anthazoa*, for review see Labas *et al*, (PNAS, (2002) 99, 4256-4261).

The cytochrome c gene was amplified by RT-PCR from a mixed human cDNA library using primers CYCS1 and CYCS2 or CYCS1 and CYCS3 according to recognised protocols (Sambrook, J. et al (2001) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press).

CYCS1; 5′-gttgaattcgaccatgggtgatgttgagaaaggc (SEQ ID NO: 11)
CYCS2; 5′-gttgttgtcgaccttactcattagtagctttttgag (SEQ ID NO: 12)
CYCS3; 5′-gttgttgtcgaccctcattagtagctttttgag (SEQ ID NO: 13)

Primer CYCS1 exhibits homology to the 5′ region of the cytochrome c gene and contains both a partial Kozak sequence (Kozak, Cell (1986), 44, 283) and an *Eco*R1 restriction enzyme site. Primer CYCS2 exhibits homology to the 3′ region of the cytochrome c gene and contains a stop codon and *Sal*I restriction enzyme site. Primer CYCS3 exhibits homology to the 3′ region of the cytochrome c gene and contains a *Sal*I restriction enzyme site. The CYCS1-CYCS2 and CYCS1-CYCS3 RT-PCR products were cloned into the corresponding *Eco*RI and *Sal*I sites of the GFP-fusion vectors pCORON1000-GFP-C1 and N1, respectively (Amersham Biosciences, Cardiff, UK) and verified by automated sequencing. These vectors contain a CMV promoter to effect the expression of the GFP fusion and an SV40 promoter to elicit expression of a neomycin resistance marker. The GFP within these vectors is red-shifted and contains the mutations F64L-S175G-E222G as described in GB 2374868.

Cytochrome c (K72A) mutants were generated with the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, Ca, USA) using primers CYCS4 and CYCS5.

CYCS4; 5′-ggagtatttggagaatcccgccaagtacatccctggaacaa (SEQ ID NO: 14)
CYCS5; 5′-ttgttccagggatgtacttggcgggattctccaaatactcc (SEQ ID NO: 15)

After sequence verification the pCORON1000-GFP-wild type cytochrome c and K72A mutant fusion constructs were sub-cloned into the vector pCORON2100 using the restriction enzymes *Nhe*I and *Not*I. pCORON2100 contains a CMV promoter and an IRES element to drive bicistronic expression of the GFP-fusion protein and a neomycin resistance marker.

The nucleic acid and amino acid sequences of the GFP-cytochrome c constructs obtained are shown in Figures 6 to 13 (SEQ ID NOS: 3-10)

Example 2

Influence of cytochrome c-K72A (APAF-1 binding) mutation upon GFP-fusion protein stable cell line generation in mammalian cells.

Plasmid DNA to be used for transfection was prepared for all constructs using the HiSpeed plasmid purification kit (Qiagen, Westberg, NL). In addition to the constructs in example 1, pCORON1000-GFP and pCORON2100-GFP were used as selection controls. DNA was diluted to 100 ng. μl^{-1} in 18-Megohm water (Sigma, Dorset, UK) and 1 µg used for transfections. For 50-80% confluency on the day of transfection, Hek293 cells were plated at a density of $5x10^4$/well in 6-well plates and incubated overnight. A 1:3 (1 µg : 3 µl) ratio of DNA to FuGene6 reagent (Roche Diagnostics, Basel, Switzerland) was used for each transient transfection reaction; 3 µl FuGene6 was added to 87 µl serum-free DMEM medium (Sigma) (containing penicillin/streptomycin, L-glutamine [Invitrogen, Carlsbad, CA]) and gently tapped to mix, then 10 µl (1 µg) construct DNA was added and again gently mixed. The FuGene6 : DNA complex was incubated at room temperature for 40 minutes and added dropwise, with gentle mixing, directly to the cells without changing the medium. The plates were then gently swirled for even distribution. Cells were monitored for expression after 24 and 48 hours using a Nikon Eclipse TE200 epifluorescent microscope (Nikon, Melville, NY). Cells were passed into 15cm diameter plates and after 24 hours placed under selection with geneticin (G418, 250 ng. μl^{-1}; Sigma). The concentration of geneticin was increased incrementally to 500 ng. μl^{-1} over the following 5-7 days. Selection continued for around 10 days or until cells in the mock-transfected control plates had died. Cloning rings were then used to isolate surviving colonies and cells were expanded through 96-well, 24-well and 6-well plates. Where appropriate second and third rounds of clonal selection were applied.

After the first round of clonal selection surviving cells were visible for transfections containing pCORON1000-GFP, pCORON2100-GFP and the pCORON2100-GFP-N and -C cytochrome c (K72A) mutant plasmids. No surviving colonies were obtained from cells transfected with other plasmid contructs.

Results

Expression studies of this mutated cDNA were performed in pCORON2100 (Amersham Biosciences) in order to utilise the IRES element and facilitate the generation of stable cell lines. A "mixed population" stably expressing cell line under selection with geneticin G418 was continuously cultured for 3 weeks. 15 single clonal cell lines were then isolated.

Hek293 cells containing the pCORON2100-GFP-cytochrome c (K72A) mutants were shown to exhibit stable expression during continuous culture over a 4 month period. The mitochondrial localisation of the reporter fusion proteins was confirmed during this period by co-localisation with the known mitochondrial marker mitotracker red (Molecular Probes, Eugene, OR) using a Microsystems LSM (Zeiss, Thornwood, NY) and a high throughput laser scanning confocal microscope (INCell Analyzer 3000, Amersham Biosciences).

The expression results from one of these stable clones (2B6), using the InCell Analyzer 3000 (Amersham Biosciences, UK) laser scanning confocal imaging system, are shown below in Figure 2. As can be seen from Figure 2a the localisation pattern of expressed cytochrome c-GFP closely resembles that observed by Goldstein et al., (Nat Cell Bio., (2000) 2, 156-160), using a confocal microscope, reproduced in Figure 2b. As expected, the fusion protein demonstrates nuclear exclusion and localises to mitochondria, the cytochrome c-GFP displaying a punctuate pattern of fluorescence (Figure 2a).

Figures 3a and 3b show localisation of the fusion protein construct (a) and the mitochondrial stain TMRE (b) at a concentration of 40nM in the mitochondria of Hek 293 cells. Dual excitation of the cytochrome c-GFP and TMRE in the InCell Analyzer demonstrates co-localisation (Figure 3c).